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# Molecular detection of carbapenem-resistant genes in clinical isolates of *Klebsiella pneumoniae*

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*Key words: K. pneumoniae, Imipenem resistance, Carbapenem-resistant, Metallo-beta-lactamase*
*Parole chiave: K. pneumoniae, Imipenem resistenza, Carbapenem resistenza, Metallo-betalattamasi*

**Abstract**

**Introduction**. *The emergence of carbapenem-resistant Klebsiella pneumoniae (CRKP) poses a considerable threat to public health worldwide. The aim of this study was to determine the prevalence of CRKP isolated from clinical specimens by phenotypic and genotypic methods.*
**Methods**. *In total, 110 consecutive non-repetitive isolates of K. pneumoniae were analyzed. Minimal inhibitory concentrations (MICs) of imipenem were determined. The mechanism of resistance was evaluated by imipenem-EDTA combined disk test and modified Hodge test. PCR method was used for the detection of blaVIM, blaIMP, blaNDM-1, blaOXA-48 and KPC genes.*
**Results**. *Totally, 8 (7.3 %) isolates were resistant to imipenem, showing MIC ≥4 μg/mL. Based on imipenem-EDTA combined disk test, all imipenem-resistant isolates were metallo-beta-lactamase (MBL) positive. PCR confirmed that 6 (75%) isolates were blaNDM-1 positive. Other resistance genes (blaVIM, blaIMP, blaOXA-48 and KPC) were not detected.*
**Conclusions**. *Based on this study, the prevalence of CRKP strains was not at a high level, however, continuous monitoring of antibiotic resistance should be performed to control dissemination of CRKP infections.*

## Introduction

*Klebsiella pneumoniae* (*K. pneumoniae*) is a Gram-negative, rod-shaped, encapsulated and non-motile bacterium that can cause different types of infections including upper respiratory tract infections (URTI), bloodstream infections, wound infections, meningitis and urinary tract infections (1). *K. pneumoniae* is one of the most important bacteria associated with health-care issues especially in patients with different risk factors including cancer, chronic liver disease, dialysis and previous antibiotic consumptions that make them more susceptible to *K. pneumoniae* infections (2, 3).

Several investigations demonstrated that some isolates of *K. pneumoniae* are hypervirulent and are linked to severe diseases such as liver abscesses, lung abscesses, endophthalmitis and meningitis, with high rate of morbidity and mortality. The capsulated serotypes, a pathogenicity island and the virulence plasmids are the best known hypervirulence-associated factors in hypervirulent isolates of *K. pneumoniae*

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(4, 5).

Treatment of *K. pneumoniae* infections has become more complicated than before. A comprehensive report on infectious diseases in European countries published by the European Centre for Disease Prevention and Control (ECDC) revealed that the trend (2008-2011) of resistance against different antibiotics such as cephalosporin, carbapenem and aminoglycosides has significantly increased in the majority of European countries (6).

Different antibiotic resistance mechanisms, especially resistance to carbapenems, have been identified in *K. pneumoniae*. The production of beta-lactamase enzymes is one of the most important mechanisms (7). According to Ambler classification, four types (A, B, C and D) of these enzymes have been identified. Based on molecular structure and protein homology these classes are divided into different subclasses. The most clinically important carbapenemase enzyme genes in carbapenem-resistant *K. pneumoniae* (CRKP) belong to Ambler class A (KPC type), class B (NDM, VIM and IMP types) and class D (OXA-48-like) (8).

Given the substantial impact of CRKP on patients' outcomes and the need of identifying resistance genes to curtail the spread of infection, the aim of this study was to determine the prevalence of CRKP isolates from clinical specimens by phenotypic and genotypic methods.

## Materials and methods

*Specimen collection and identification*

In total, 110 consecutive non-repetitive isolates of *K. pneumoniae* were collected from patients referring to Sistan and Balouchestan hospitals. Standard biochemical tests including Gram-stain, catalase, and lactose fermentation were used for primary identification. Species-specific PCR was carried out using previously designed primers for ITS (16s–23s rRNA internal transcribed spacer) (9, 10).

*Antimicrobial susceptibility testing*

Resistance to ertapenem (ETP, 10 μg), meropenem (MEM, 10 μg), ciprofloxacin (CIP, 5 μg), amikacin (AMK, 30 μg), gentamicin (GM, 10 μg), cefepime (CPM, 30 μg), cefoxitin (FOX, 30 μg) and ceftriaxone (CRO, 30 μg) purchased from MSAT, Merseyside, UK, was evaluated by Kirby-Bauer's disk diffusion method (11). Minimal inhibitory concentrations (MICs) of imipenem were determined according to CLSI guidelines and broth microdilution method (11). The concentration gradient for imipenem (Sigma-Aldrich, USA) was 64-0.5 μg/mL. *K. pneumoniae* isolates that were resistant to imipenem were subjected to phenotypical detection of metallo-beta-lactamase enzymes by imipenem-EDTA combined disk test as described earlier (12). The modified Hodge test (MHT) was performed based on CLSI recommendations. Briefly, standard suspension of *E. coli* 25922 was prepared and inoculated into Muller-Hinton agar. A 10 μg ertapenem disk (Mast, UK) was placed in the center of the plate. Suspected isolates were inoculated in straight line out from the edge of the disk. Finally, after an overnight incubation period, the presence of a "cloverleaf shaped" inhibition zone was considered positive (11).

*Molecular detection of resistance genes by polymerase chain reaction (PCR)*

Genomic DNAs were extracted by boiling method (13). Briefly, two fresh colonies of *K. pneumoniae* were suspended in 300 μL sterile distilled water. After complete dissolving, the suspension was heated for 10min at 100$^{o}$ C. Finally, after cooling at room temperature, the suspension was centrifuged at 13,000g for 10 min and the supernatant was used as a source of DNA template for amplification. PCR amplification

was carried out on extracted DNAs for the detection of *bla*VIM, *bla*IMP, *bla*NDM-1, *bla*OXA-48 and *KPC* genes using specific primers (14). All PCR amplifications were performed by using Ampliqon (Denmark) ready to use master mix. The following program was used: initial denaturation at 94° C for 5 min, 30 cycles of denaturation at 94° C for 1 min, annealing at 51° C for 1 min, extension at 72° C for 50 sec and a final extension at 72° C for 10 min. PCR products were separated by electrophoresis on 1% agarose gel and were stained with Sybr safe (Thermo Fisher Scientific Inc., USA). PCR products were sent to Macrogen (South Korea) company for purification and sequencing on both strands. We used Mega6 as a multiple sequence alignment software and blast website (https://blast.ncbi.nlm.nih.gov/Blast) for sequence analyzing (15).

## Results

One hundred and ten isolates of *K. pneumoniae* were obtained from sputum 51 (46.4%), urine 38 (34.5%), blood 12 (10.9%), wound 5 (4.5%) and other clinical specimens (trachea, synovial fluid and abscess) 4 (3.6%). From these, 49 (44.5%) were female and 61 (55.5%) were male. Meanwhile, out of 110 isolated bacteria 57 (51.8%) were from hospitalized patients and 53 (48.2%) were from outpatients. The highest susceptibility was observed against ertapenem (n = 102; 92.7%) and meropenem (n = 102; 92.7%), followed by amikacin (n = 100; 90.9%), gentamicin (n = 97; 88.2%), cefepime (n = 97; 88.2%), ciprofloxacin (n = 74; 67.3%), cefoxitin (69; 62.7%) and ceftriaxone (n = 69; 62.7%). Totally, 8 (7.3%) isolates were resistant to imipenem, with MIC ≥4 μg/ml (Table 1). Other isolates showed MIC ≤1. Imipenem-resistant isolates were resistant to all antibiotics except to amikacin. Based on imipenem-EDTA combined disk test, all imipenem-resistant isolates were MBL positive. Meanwhile, modified Hodge test was positive in 2 imipenem-resistant isolates. PCR confirmed that 6 (75%) isolates contained *bla*NDM-1 (Figure 1). Other resistance genes (*bla*VIM, *bla*IMP, *bla*OXA48 and *KPC*) were not detected. Results of blast with other sequences deposited in GenBank database revealed 99-100% similarity with *bla*NDM-1. Determined nucleotide sequences were submitted to GenBank under following accession numbers; MH102400, MH102401 and MH120290.

Table 1 - Characteristics of eight imipenem-resistant *K. pneumoniae*

| Isolate ID | Sex | Source of isolation | Hospitalization (ward) | IPM MIC (μg/ml) | MBL | Resistance gene |
|---|---|---|---|---|---|---|
| A33 | Female | Sputum | Yes (Emergency) | 4 | Positive | *bla*-NDM-1 |
| A35 | Male | Sputum | Yes (Emergency) | 64≤ | Positive | *bla*-NDM-1 |
| A338 | Female | Urine | Yes (ICU) | 16 | Positive | *bla*-NDM-1 |
| A387 | Female | Sputum | No | 16 | Positive | *bla*-NDM-1 |
| A126 | Female | Sputum | No | 16 | Positive | *bla*-NDM-1 |
| A134 | Female | Sputum | Yes (Emergency) | 16 | Positive | *bla*-NDM-1 |
| A6 | Male | Wound | Yes (ICU) | 4 | Positive | Negative |
| A7 | Female | Urine | No | 4 | Positive | Negative |

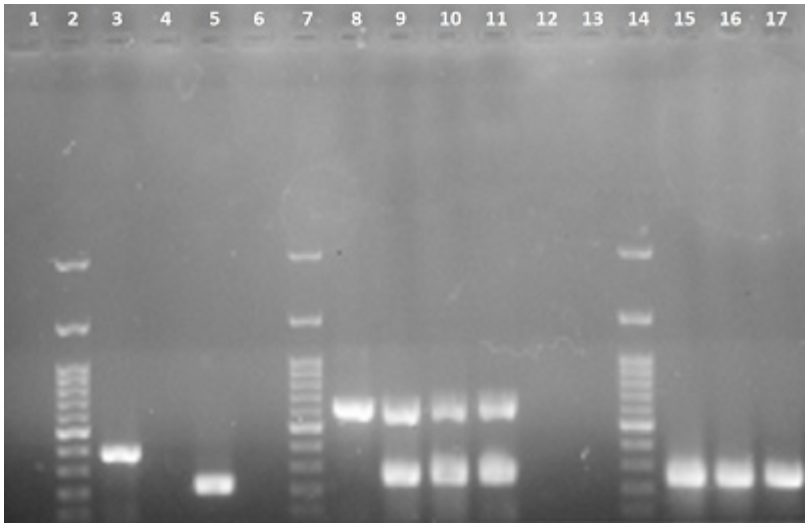


Figure 1 - Agarose gel electrophoresis used for PCR product separation, lines 2, 7, and 14, 100bp ladder; lines 1, 4, 6, 12, and 13 negative control; line 3, *bla*-VIM (390 bp) positive control; line 5, *bla*-IMP (232 bp) positive control; line 8, *bla*-NDM (621 bp) Positive control; lines 9, 10, and 11, clinical NDM positive isolates; lines 9, 10, 11, 15, 16, and 17, positive species-specific PCR (260 bp)

## Discussion

The use of antibiotics plays an important role in the treatment of infections caused by drug-susceptible microorganisms. However, the effectiveness of antibiotic therapy has been reduced owing to different mechanisms of antibiotic resistance. The emergence of drug-resistant infections is considered to be a serious challenge to human health worldwide. In fact, widespread use and misuse of antibiotics led to a decline in the effectiveness of traditional antimicrobial therapy and appropriate antibiotic selection (16).

Carbapenems are among the main members of the beta-lactam antibiotics and are being used for treatment of infections caused by bacteria showing resistance against multiple antibiotics including penicillin and cephalosporins. Furthermore, studies revealed that CRKP infection is linked to high rate of mortality, morbidity, and health-care costs (17). Therefore, determining the prevalence of CRKP isolates is completely necessary.

Given the great impact of CRKP on patients' outcomes and the availability of limited informations in our region, this study was conducted to evaluate the prevalence of CRKP in Sistan and Balouchestan province.

Our findings revealed that the prevalence of CRKP was 7.3%. Results of different independent studies in Iran showed that the prevalence of CRKP is variable. For example, in a study from Tehran, Bina reported that 37% of investigated *K. pneumoniae* isolates were CR (18), while other studies conducted in Isfahan province demonstrated 0% resistance against carbapenem (19, 20). Additionally, in comparison with European countries, our findings are much lower than Romania (24.7%), Italy (33.5%), and Greece (61.5%). However, reported prevalence is similar to Bulgaria (7.2%) and Malta (9.9%) (21).

In this study, out of 8 carbapenem resistant isolates, 6 isolates were confirmed as MBL positive. On the other hand, in two isolates with positive imipenem-EDTA reaction, investigated MBL genes were not detected. This discrepancy might be

related to other MBL genes or carbapenem resistance mechanisms such as Ambler class D beta-lactamases, mutation in outer membrane porins or efflux pump over-expression. Additionally, it has been shown that bactericidal activity of EDTA may lead to false positive results once phenotypic methods are being applied (22, 23).

Based on our results, *bla*NDM-1 gene is responsible for carbapenem resistance. For the first time, this gene was found in a *K. pneumoniae* isolated from urine culture of a male patient in Sweden who had been hospitalized in India (24). Following the first report of NDM in 2009, infections caused by NDM producer isolates have been reported from many other countries such as Vietnam, Netherlands, Serbia, Pakistan, Japan, Guatemala, Australia and the USA, indicating rapid and widespread dissemination (24). Since transferable plasmids harboring *bla*NDM often contain other antibiotic resistance genes, infection caused by NDM producer strains must be considered as a serious threat to patients' health (25).

This is the first report of *bla*NDM-1 positive *K. pneumoniae* in Sistan and Balouchestan province. In Iran, *bla*NDM producer isolates have been previously reported from Isfahan and Tehran provinces. Shahcheraghi et al. during a study on 45 clinical isolates of *K. pneumoniae* collected from Tehran hospitals, reported detection of *bla*NDM-1 (26). Likewise, Fazeli et al., in a study performed on 112 clinical isolates of *K. pneumoniae* collected from urine, sputum, wound, cerebrospinal fluid, bronchoalveolar-lavage (BAL) and abscess between 2012 and 2013, reported the presence of *bla*NDM-1 among carbapenem-resistant isolates (27).

Our results revealed that only two isolates of imipenem-resistant strains were MHT positive. This finding was in accordance with other reports. For instance, it has been shown that the overall sensitivity of MHT was low due to high rate of false negative results among *bla*NDM producer isolates (28, 29).

The detection of *bla*NDM harboring strains is of paramount importance because these isolates are often resistant against different antibiotics, resulting in limited available treatment options. Moreover, these resistance genes are often located at the transposable genetic elements such as plasmids and integrons; therefore, they can spread easily between bacteria by horizontal gene transfer (24, 25).

## Conclusions

Infections caused by CRKP are considered to play a significant role in the treatment failure and poor patients' outcomes. Therefore, continuous surveillance of the emergence of carbapenem-resistant isolates should be considered as a very important issue in the microbiology laboratories. In the present study, the prevalence of CRKP strains was not at a high level, however, constant monitoring of MBL-producing isolates is necessary in order to detect in real time the emergence of multi drug resistance (MDR) isolates and possibility of dissemination of resistance genes. Furthermore, phenotypic detection method by imipenem-EDTA was an accurate and simple method which can be used as a reliable detection method in all microbiology laboratories at any level.

Although in this study the most important genes conferring resistance to carbapenems were investigated, in order to prevent the spread of resistant strains other resistance mechanisms including efflux pump over-expression, porin loss, and other beta-lactamase genes will also be studied in the future.

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## Riassunto

### Identificazione molecolare dei geni della carbapenem-resistenza in ceppi di isolamento clinico di *Klebsiella pneumoniae*

**Premessa**. La comparsa di ceppi di *Klebsiella pneumoniae* carbapenem-resistenti costituisce un considerevole pericolo per la Sanità Pubblica a livello mondiale. Scopo del presente studio è di determinare con metodi fenotipici e genotipici la prevalenza in campioni clinici dei ceppi di *K. pneumoniae* carbapenem-resistenti.

**Metodi**. In totale sono stati esaminati 110 isolamenti consecutivi non ripetitivci di *K. pneumoniae,* determinandone le minime concentrazioni inibenti (MICs) di imipenem. Il meccanismo della resistenza è stato valutato con il test del disco combinato imipenem-EDTA e con il test di Hodge modificato. Il metodo PCR è stato usato per evidenziare i geni *bla*VIM, *bla*IMP, *bla*NDM-1, *bla*OXA-48 e *KPC*.

**Risultati**. Nell'insieme, 8 (7,3%) isolamenti sono risultati imipenem-resistenti, con un valore di MIC ≥4 μg/mL. Sulla base del test del disco combinato imipenem-EDTA, tutti gli isolamenti imipenem -resistenti sono risultati anche postivi alla metallo-beta-lattamasi (MBL). Il test PCR ha confermato che 6 (75%) degli isolamenti erano *bla*NDM-1 positivi. Non sono stati rinvenuti altri geni di resistenza (*bla*VIM, *bla*IMP, *bla*OXA-48 and *KPC*).

**Conclusioni**. Sulla base del presente studio la prevalenza dei ceppi CRKP non è risultata di frequenza elevata, tuttavia un monitoraggio continuo della resistenza antibiotica deve essere mantenuta per controllare la disseminazione delle infezioni CRKP.


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